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The T-box family of genes play critical roles in inductive interactions in the development of many organs. Two of these genes, *Tbx2* and *Tbx3*, are expressed in the developing mammary glands of mice and one is in adult glands and mammary tumors. Our hypothesis is that *Tbx2* and *Tbx3* are required in the signalling pathway of normal mammary gland development, and could be involved in mammary carcinogenesis. Previously, we proposed *TBX3* as a candidate gene for the Ulnar-mammary Syndrome in humans, based on the observed expression patterns and the chromosomal location of the locus, and this has recently been proven to be the case. Mutations in this gene are responsible for the mammary hypoplasia characteristic of this syndrome. Our goals are to explore the roles of *Tbx2* and *Tbx3* in mammary development and carcinogenesis by targeted mutagenesis of these genes. In work to date we have detailed the expression patterns and have begun produced null mutations in each gene. The significance of this work is in elucidating normal mechanisms of mammary development and thereby aiding in the understanding of how growth controls can go wrong in cancer.

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INTRODUCTION

A vital missing link in understanding the ontogeny of breast cancer is the identification and investigation of genes controlling normal mammary gland development during embryonic organogenesis and during pregnancy and lactation. We are investigating a newly discovered family of putative transcription factors, called the T-box family, that is thought to play critical roles in controlling inductive interactions in the development of many organs, including the mammary glands.

The T-box gene family is defined by a region of homology to the DNA binding domain of the mouse *Brachyury*, or *T* locus gene product. Phylogenetic studies have demonstrated the ancient origin of this gene family. At least 16 different T-box genes have now been identified in the genomes of animals ranging from *C. elegans* to humans (Papaioannou and Silver, 1998). In the mouse, we have identified 6 T-box genes that are expressed in highly specific patterns during early embryogenesis and organogenesis, in areas of classic embryonic inductive interactions (Chapman *et al.*, 1996). Two of these genes, *Tbx2* and *Tbx3*, are expressed in the developing mammary glands, and one of them is expressed in adult glands and in mammary tumors (our unpublished data). In the embryo, they are expressed reciprocally in the mesenchymal and epithelial components of the gland, suggesting they work in concert during the branching of the epithelial ducts.

Our hypothesis is that *Tbx2* and *Tbx3* are required in the signalling pathway of normal mammary gland development, and could also be involved in neoplasia. Previously, we identified *Tbx3* as a candidate gene for the Ulnar-mammary Syndrome in humans, based on the observed expression patterns and the chromosomal location of the locus (Papaioannou, 1997). This has recently been proven to be the case (Bamshad *et al.*, 1997), and mutations in this gene have been shown to be responsible for the mammary hypoplasia characteristic of this syndrome. Our goals are to explore the roles of *Tbx2* and *Tbx3* in mammary development and mammary carcinogenesis using a mutational approach.

BODY

Experimental Methods

Gene Expression

Mammary glands were dissected from adult virgin, pregnant, lactating, and involuting female mice, and from a mammary tumor dissected from a MMTV-*neu* transgenic mouse. RNA was isolated (Chomczynski and Sacchi, 1987) and used for Northern analysis. Hybridization of *Tbx2* and *Tbx3* probes was performed at 65°C. Embryos were dissected at various gestational ages and sexed by the gross morphology of the gonads or by staining for sex chromatin in the amnion. Embryos were then subjected to whole mount *in situ* hybridization with probes specific for *Tbx2* and *Tbx3* (Chapman *et al.*, 1996).

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Mammary Gland Outgrowth and Antisense Experiment

Mammary gland primordia were dissected out as epithelia buds from 13.5 and 14.5 dpc embryos by gross morphology (Sakakura, 1987) and the donor embryos sexed by gonadal morphology. Gland explants were grown in organ culture (Kratochwil, 1969) on Transwell filters floated on media consisting of 10% fetal calf serum in DMEM for up to seven days at 37°C in 5% CO_2 in air. Gland anlagen were classified according to sex, age of embryo at time of explantation, axial or inguinal location, and degree of development attained. After 13.5 dpc, axial anlagens (from both male and female embryos) were determined to have the highest rate of successful development, and these were used exclusively for further experimentation. For antisense experiments, glands were cultured as above or with media supplemented with phosphorothioate oligonucleotides (S-oligos). Glands incubated with antisense S-oligos against *Tbx3* or with the sense control were compared to contralateral glands grown without oligos.

Gene Targeting

Mouse *Tbx3* genomic clones were isolated from a genomic λ phage 129/Sv library (Stratagene). The targeting construct was generated by replacing a 3.2 kb *SpeI* fragment with a *lox*P flanked selection cassette containing the herpes simplex virus thymidine-kinase gene under the control of its own promoter and the neomycin-resistance gene under the control of the phosphoglycerate kinase promoter. The diphtheria toxin gene under the control of the β-actin promoter (Maxwell *et al.*, 1987) was placed 5' of the upstream homology for negative selection, and the construct was linearized at a unique *Not*I site. The linearized construct was electroporated into R1 ES cells (Nagy *et al.*, 1993). We isolated DNA from cell clones, aggregated cells with morulae, and injected cells into blastocysts according to standard protocols (Joyner, 1993).

RESULTS

Expression of Tbx2 and Tbx3

We have used standard Northern analysis of mRNA isolated from mammary glands of normal adult mice and standard whole mount *in situ* hybridization analysis of embryonic mice at different days of gestation. Using these methods, we have detected *Tbx3* expression in virgin, pregnant, lactating and involuting mammary glands of adult mice (Figure 1), although *Tbx2* is not expressed in these adult tissues nor in mammary tumors (data not shown). At the onset of mammary development in the midgestation embryo, *Tbx2* is expressed in the mesodermal stroma or milk line, underlying the mammary epithelial buds, although this expression is not evident beyond the 12th day of gestation. *Tbx3*, on the other hand, is expressed in the epithelial buds of both male and female embryos through the 15th day of gestation (Table 1).

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Table 1. Expression of *Tbx2* in stroma and *Tbx3* in epithelium of developing mammary glands in mouse midgestation embryos of both sexes.

		EXPRESSION	
Age (days post coitus)	Sex	*Tbx2* (stroma)	*Tbx3* (epithelium)
11.5	-	+	-
12.5	♀	-	
	♂	-	+
13.5	♀	-	+
	♂	-	+
14.5	♀	-	+
	♂	-	+
15.5	♀	n.d.	-
	♂	n.d.	-
16.5	♀	-	-
	♂	-	-

Mammary Gland Organ Cultures

Although not a part of our original proposal, we have begun culturing mammary gland primordia in order to develop a culture system in which to examine branching morphogenesis. We have successfully established an in vitro culture system for embryonic mammary glands which recapitulates normal gland development. Using gland primordia dissected from 13.5 dpc embryos, as high as 60% of glands developed branching morphogenesis (2-15 branch tips visible). Both sense and antisense phosphorothioate oligos (S-oligos) directed against Tbx3 inhibited development of the gland anlagen at concentrations at or above 5 micromolar, but neither affected development below this concentration. We concluded that the effects of the S-oligos were nonspecific at quite low concentrations and any future attempts at antisense manipulation will require a delivery vehicle enabling lower concentrations of oligo to be used. However, the development of the embryonic mammary gland culture system in this lab will allow us to perform other *in vitro* manipulations of the developing mammary gland, including tissue recombination experiments with *Tbx2* and *Tbx3*

mutant tissue.

Gene Targeting

Because *Tbx2* and *Tbx3* are expressed in several embryonic tissues in addition to the mammary glands (Chapman *et al.*, 1996), mutations in either gene are likely to have multiple effects on embryogenesis. Although we have initially produced null mutations in both these genes as part of our general program investigating the T-box gene family, our eventual aim for this project is to produce mammary-specific loss of function through conditional targeted mutagenesis using the Cre/*loxP* system. Toward this aim, we have characterized the *Tbx3* gene and its transcripts in order to make an appropriate targeting construct. Northern blot analysis of adult and embryonic mRNA indicates two predominant transcript sizes, 4.5 and 5.2 kb (Bollag *et al.*, 1994). A number of genomic clones have been restriction mapped, aligned and subcloned (Figure 2). Together these clones cover ~20 kb of the *Tbx3* genomic locus including the T-box which codes for the DNA binding domain.

A targeting construct was made from these genomic clones that replaces almost the entire coding region of the T-box with a *lox*P flanked selection cassette, resulting in a null mutation in *Tbx3* (Figure 3). We also plan to use these genomic clones to make a similar construct that will leave the coding sequence intact, but flank the T-box with *lox*P sites for mammary-specific, conditional gene targeting (Figure 4). The targeting construct producing a null mutation was used for three electroporations into R1 ES cells. Seven targeted clones were identified from 114 screened ES clones. Two of these targeted cell lines have been used to generate chimeras by either aggregation with Black Swiss morulae or cell injection into C57BL/6 blastocysts. Recently, three injection chimeras from one of the targeted cell lines were identified as germline transmitters of the targeted mutation and heterozygous mice are currently being generated by breeding these animals. Heterozygous offspring will be carefully examined for phenotypes present in humans with ulnar-mammary syndrome, including mammary hypoplasia and forelimb deficiencies. If the mouse gene has a dose dependency similar to the human, we expect that these animals will provide models for examining the mammary hypoplasia characteristic of the ulnar-mammary syndrome.

Related work in this laboratory and future plans

We have also been successful in producing a null mutation in the *Tbx2* gene, which, has the potential of being used to produce a conditional, tissue-specific mutation. This project is at an early stage of analysis and no results of the null mutation phenotype are available as yet. However, the availability of these mutant animals provides us with the opportunity to interbreed the mutants to produce double mutants. Eventually we should also be able to use the null mutant animals for both *Tbx3* and *Tbx2* to make use our mammary gland *in vitro* outgrowth system to recombine tissues of different genotypes to investigate potential tissue interactions during mammary gland development.

CONCLUSIONS

Work in other laboratories has now identified mutations in *Tbx3* as the causal factor in the ulnar-mammary syndrome in humans, vindicating our interest in this gene as a controlling gene in the normal development of the mammary gland. We have made progress in identifying areas of expression of the genes in the developing and adult mammary glands. We have also made progress towards molecular characterization of the gene. The major advance in the final year of this two year exploratory grant period was in the construction of a gene targeting vector and the successful production of targeted ES cells and eventually, mice carrying the targeted mutation. These animals have a essentially a null mutation in Tbx3, and are thus genetically similar to the human ulnar-mammary syndrome patients. Continuation of the project will include a phenotypic characterization of the null mutations for both *Tbx2* and *Tbx3* in heterozygous and homozygous animals, production of mammary specific mutations in these genes making use of Cre/*lox*P technology, if warranted, and use of the mammary primordia *in vitro* outgrowths to investigate tissue interactions during mammary gland development.

This two year exploratory grant has been extremely valuable in allowing us to begin investigation of a new gene based on predictions and very preliminary evidence that it would be involved in mammary gland development. During this period, our work and other developments in the field have identified this gene as a prime player in early development of the mammary gland and so our predictions were vindicated. The progress we made in creating a mouse model for a human syndrome puts us in an excellent position to make rapid advances toward the elucidation of the gene's role.

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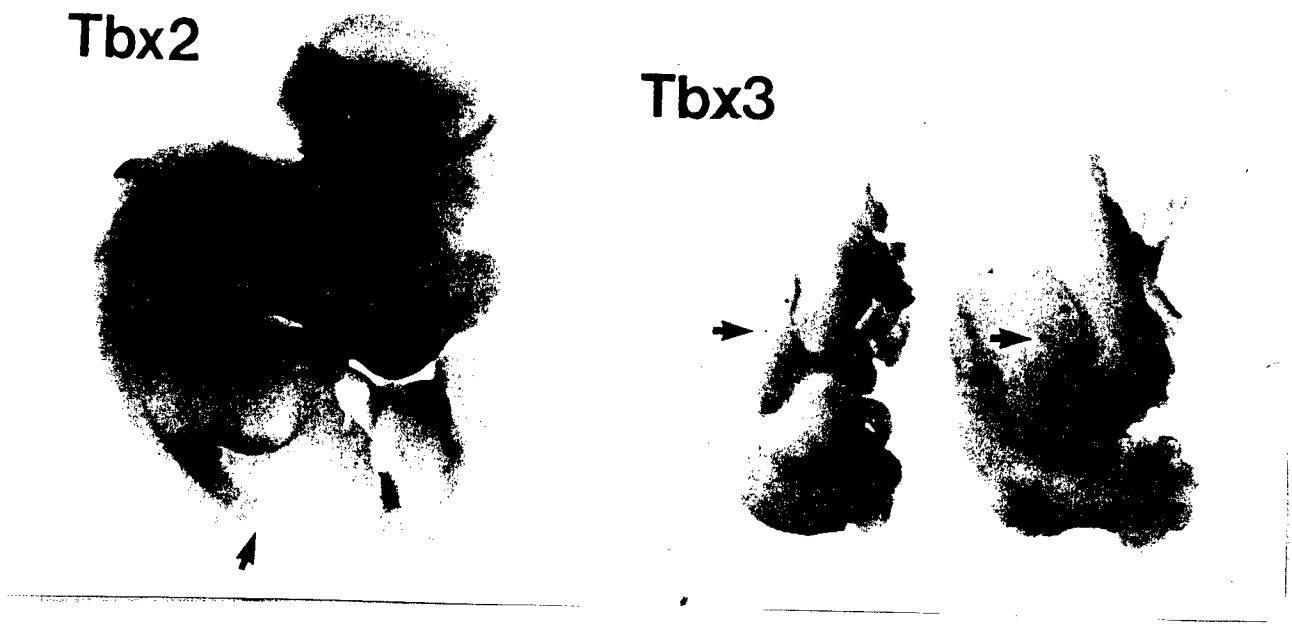
Figure 1.

Whole mount *in situ* hybridization analysis of *Tbx2* and *Tbx3* expression in the mid-gestation embryo. *Tbx2* is expressed as a stripe (arrow) along the lateral body wall extending from the anterior to posterior limb bud in the day 11.5 dpc embryo. *Tbx3* expression is detected as discrete spots (arrow) in the lateral body wall of the day 13 and 13.5 dpc embryos, which correspond to the epithelial buds of the developing mammary gland.



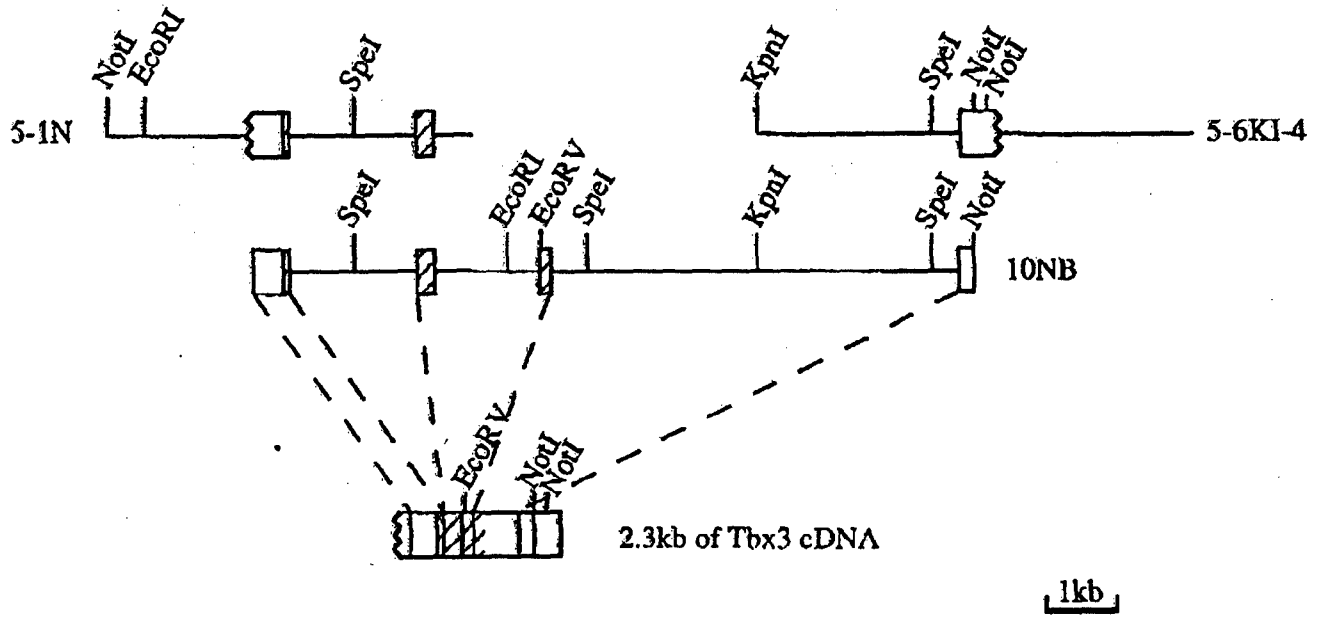


Figure 2. *Tbx3* genomic clones aligned with *Tbx3* cDNA. Three genomic clones, p5-1N, p5-6KI-4, and p10NB, were cloned into plasmid from three independent genomic phage clones and aligned by restriction mapping. Southern hybridization and DNA sequencing were used to map exons. Hatched region indicates the T-box.

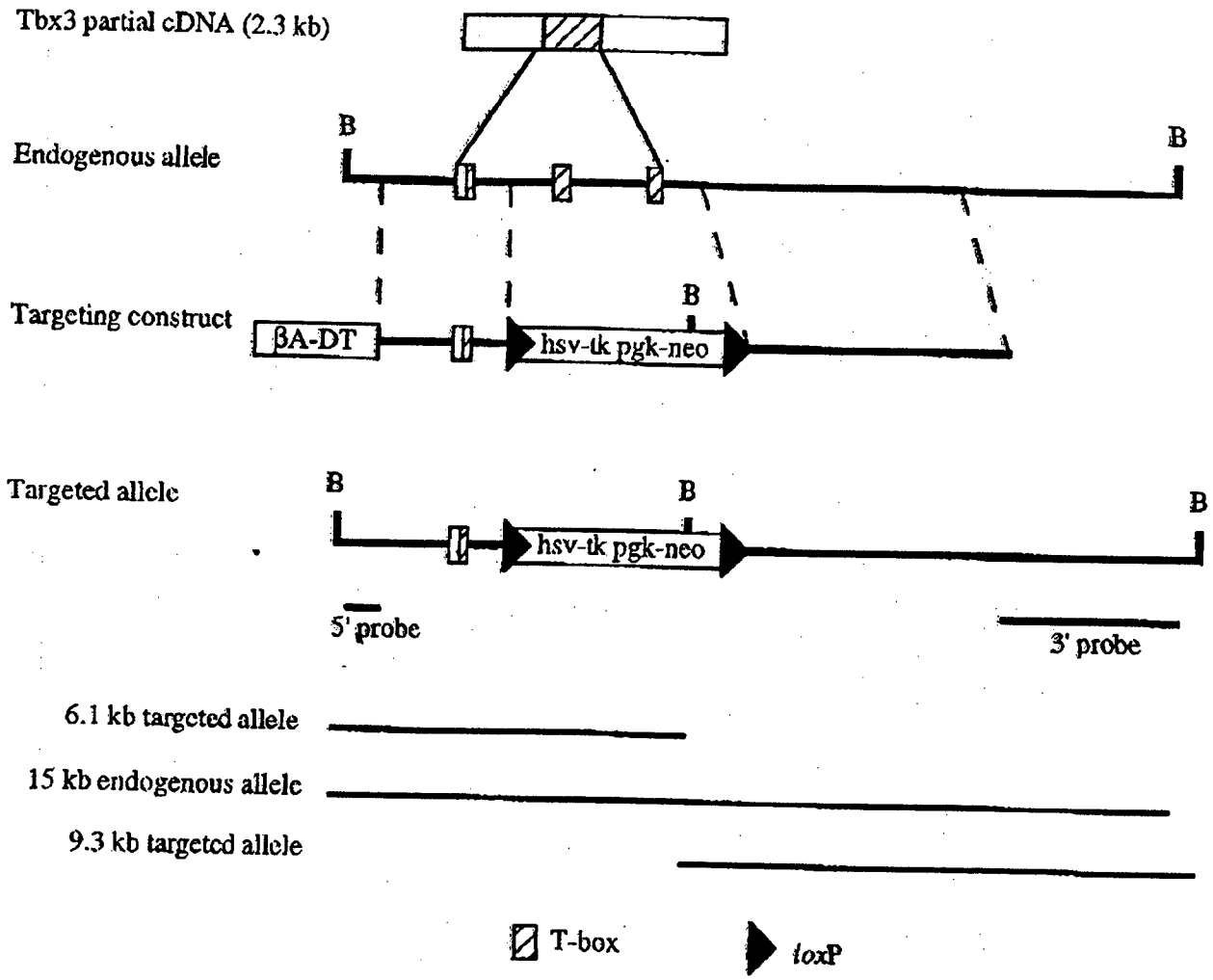


Figure 3. Targeting strategy for *Tbx3* showing the endogenous genomic allele, the targeting construct, and the targeted allel with resulting diagnostic digests. The targeting construct contains negative selection DT (diphtheria toxin), positive selection neo (neomycin resistance), and *lox*P sites flanking the selection cassette to allow removal of the insertion with the Cre recombinase and tk (thymidine kinase) negative selection.

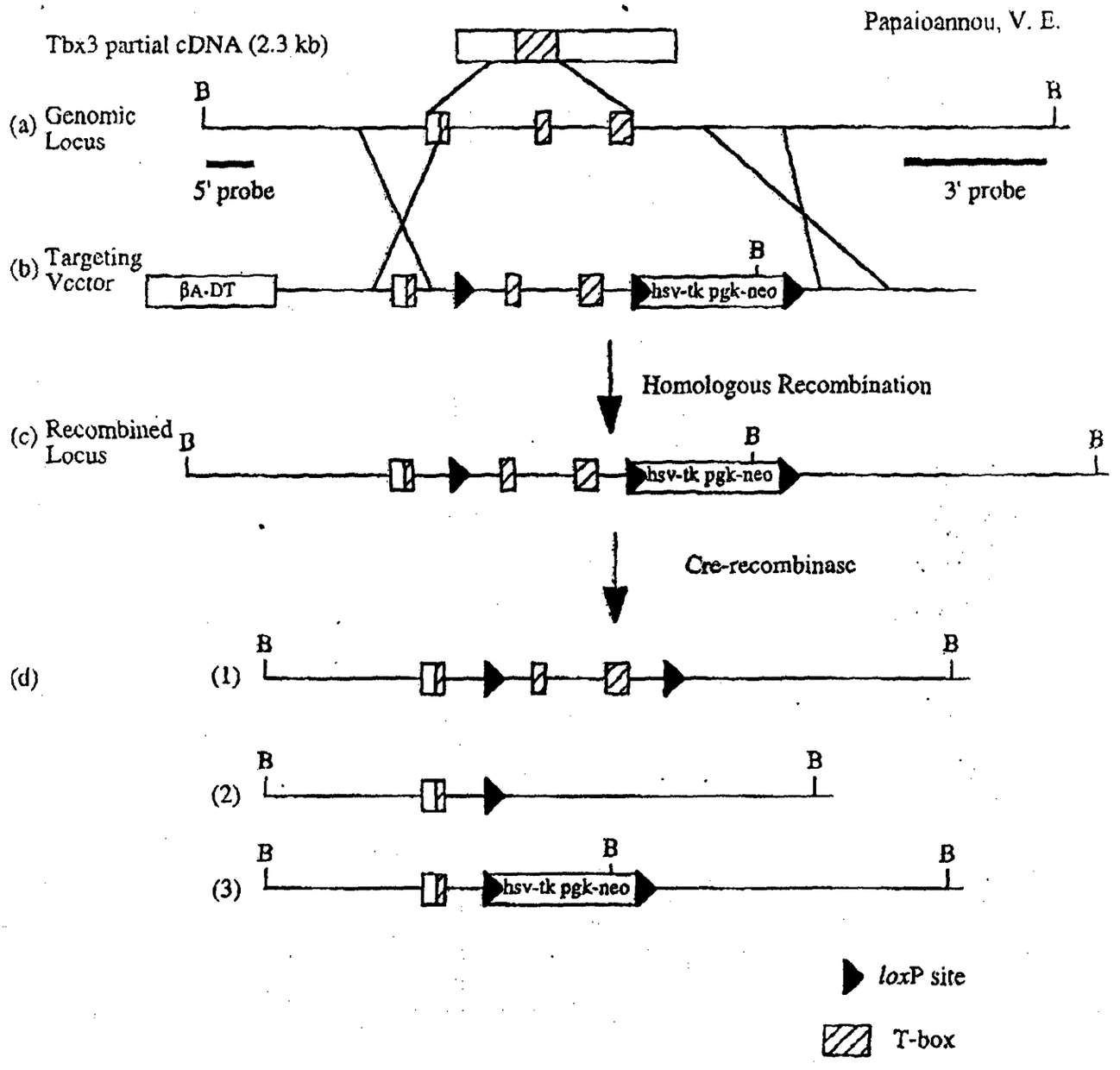


Figure 4. Mammary-specific conditional gene targeting strategy. Homologous recombination between the genomic locus (a) and the targeting vector (b) introduces three *lox*P sites into the recombined locus (c). Cre recombinase catalyzes recombination between the *lox*P sites to produce three possible products (d). The firest product leaves the gene intact, but flanks the T-box with *lox*P sites. The second product deletes the T-box. The third is selected against with gancyclovir. The *lox*P flanked T-box of the first product can be deleted in mammary glands *in vivo* by mating with a mouse carrying a *Cre* recombinase transgene under a mammary-specific promoter.

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Virginia E. Papaioannou, Ph.D.
Deborah L. Chapman, Ph.D.

Rec'd 1/11/2000

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